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(54) Title: SEX-ASSOCIATED MEMBRANE PROTEINS AND METHODS FOR INCREASING THE PROBABILITY THAT OFFSPRING WILL BE OF A DESIRED SEX

(57) Abstract

A method of sorting living cells based on DNA content. Mammalian sperm subpopulations enriched in X- or Y-sperm. X- and Y-enriched sperm-plasma-membrane vesicles. Substantially pure sex-associated membrane (SAM) proteins. Antibodies binding to X- or Y-SAM proteins, essentially free of antibodies binding to Y- or X-SAM proteins respectively, or to the H-Y antigen. Semen samples enriched for X- or Y-sperm. Methods for increasing the probability that offspring will be male or female comprising the step of allowing a sperm from an enriched semen sample to fertilize an ovum. Methods for increasing the probability that offspring will be male or female comprising the step of immunizing a female with X- or Y-SAM proteins. Methods of decreasing fertility comprising the step of immunizing a female with both X- and Y-sperm. Methods of increasing the probability that mammalian offspring will carry a gene for a particular sex-chromosome linked trait.

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DESIGNATIONS OF "DE"

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SEX-ASSOCIATED MEMBRANE PROTEINS AND
METHODS FOR INCREASING THE PROBABILITY
THAT OFFSPRING WILL BE OF A DESIRED SEX

5   FIELD OF INVENTION

        The field of this invention is the isolation
of novel proteins and their use in methods to increase
the probability that mammalian offspring produced by
it will be of a desired sex or carry a gene for a
10   particular sex-chromosome linked trait. This inven-
tion relates to sorting of sperm cells into X-enriched
and Y-enriched subpopulations. It further relates
to the isolation of X-enriched and Y-enriched sperm
plasma membranes and components thereof. More par-
15   ticularly, this invention relates to sex-associated
membrane proteins and to antibodies which bind to
them. It relates to the use of these antibodies to
modify a semen sample so that the semen sample will
be enriched in X-chromosome bearing sperm cells or
20   Y-chromosome bearing sperm cells.

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BACKGROUND OF INVENTION

Mammalian semen contains approximately equal
numbers of Y-chromosome bearing sperm cells (Y-sperm)
and X-chromosome bearing sperm cells (X-sperm). Fer-
5   tilization of an ovum by a Y-sperm produces a male.
Fertilization by an X-sperm produces a female.

Various methods have been proposed for
modifying mammalian semen to increase the relative
percentage of X- or Y-sperm in a semen sample, and
10 thereby achieve a greater likelihood of female or male
offspring. Attempts to influence or control mammalian
sex have not been verifiable. (For reviews of prior
research, see Garner, 1984; Pinkel et al., 1985.)

One of the more common approaches for
15 attempting X-sperm or Y-sperm enrichment in semen
has relied on motility and density sedimentation.
(See Kaiser et al., 1974, or Soupart, 1975.) This
approach is based on the Y-sperm's purported greater
motility and lighter weight than X-sperm. According
20 to the theory, Y-sperm would penetrate an interface
created at two different media densities more easily
than X-sperm. One such approach used albumin gradient
sedimentation. However, due to the morphological
variability of the maturing sperm, no one has
25 independently shown that this technique can separate
or enrich X-sperm or Y-sperm (Brandriff et al., 1986).

Immunological methods have also been tried
as a means of separating X- and Y-sperm. These
methods are based on the fact that spermatid RNA
30 polymerase is capable of transcribing the haploid
genome (Moore, 1971). It was believed that X- and
Y-sperm could be separated immunologically on the
basis of the different antigens produced from this
RNA transcript. Antigens investigated in unsexed
35 sperm included the LDH isozyme (Stambaugh and

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Buckley, 1971). Again, no demonstrable separation
has been reported.

Investigators have also looked to the male
H-Y antigen as a potential means to enrich sperm
5   subpopulations and thereby preselect sex of offspring.
Indirect evidence suggested that H-Y antigen was a
cell-surface antigen produced in males but not in
females. Accordingly, investigators have reasoned
that H-Y is expressed by cells containing a Y
10   chromosome and, therefore, on the surface of Y-sperm
but not X-sperm. Consequently, some investigators
believed that H-Y antibodies should inactivate Y-sperm
but not X-sperm. Some investigators have claimed to
skew mammalian sex ratios using methods based on
15   this theory (McCormick et al., 1983; Boyce and Bennet,
1984). Bryant, in particular, has claimed a dramatic
skewing of sex ratio using H-Y antibodies (Bryant,
U.S. Patent No. 4,191,749; Bryant, U.S. Patent No.
4,448,767). However, experience has not borne-out
20   these claims. Hoppe and Koo stated specifically
that they were unable to skew sex ratio using anti-
bodies against the H-Y antigen (Hoppe and Koo, 1984).
As far as we know, no one has confirmed Bryant's
claims.
25   There may be two reasons for the failure to
confirm these results. First, the underlying theory
appears to be wrong. The most recent evidence
indicates that there is no difference in H-Y presence
on mature X- or Y-sperm. While certain male tissues
30   produce H-Y and express it as an integral membrane
protein, Y-sperm do not appear to produce H-Y them-
selves. Rather, both X-and Y-sperm adsorb it to
their surface (Garner, 1984). Hoppe and Koo have
shown that both X- and Y-sperm react with H-Y anti-
35   body (Hoppe and Koo 1984). Our own evidence, which
we present herein, corroborates this. Furthermore,

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Hoppe and Koo showed that as sperm mature, their
ability to react with H-Y antigen declines, implying
that H-Y is masked or lost from the sperm cell
surface (Id.). Second, the experimental technique of
5   some of these investigators may have been flawed.
They based their conclusions on experiments with a
limited number of animals, so that the sex ratios,
while skewed, were not statistically significant
(Moore and Gledhill, 1988). Therefore, there is no
10 longer any reason to believe that one could success-
fully use the H-Y antigen to separate X-sperm and
Y-sperm. Indeed, we are not aware of any methods
currently in use which successfully use this strategy.

Fabricant et al., U.S. Patent No. 4,722,887,
15 refers to a method for separating X-and Y-sperm by
polymeric phase separation based on differential
expression of a sperm cell-surface sulfoglycolipid
(SGG). However, the authors state that the evidence
for sex-linked differences in this lipid is
20 indirect -- it is based on the sex-linked expression
of enzymes which metabolize lipid substrates -- and
the authors expess only the expectation that SGG,
itself, will prove to be sex-linked.

Another potential separation approach for
25 X-sperm and Y-sperm is based on the known differ-
ences in the DNA contents of X-sperm and Y-sperm.
Because the DNA content of X-sperm cells is
greater than the DNA content of Y-sperm cells,
investigators hoped that the respective live cell
30 populations could be separated by density gradient
sedimentation or flow cytometry. However, neither
has proven to be possible.

One reason for this failure may be that
the DNA content differences between X-sperm and
35 Y-sperm are small. For example, the difference is

believed to be only about 3.9% for bulls, 3.7% for
boars and 4.1% for rams (Sumner 1971; Pearson,
et al., 1973; Evans et al., 1972; and Gledhill,
1985). This translates into an approximate 0.003

5   difference in bouyancy -- not enough to permit
separation of whole sperm using available methods.
While other mammals display somewhat higher differ-
ences in the relative DNA contents of X-sperm and
Y-sperm, e.g., the vole (Microtus oregani) which has

10   about a 9% difference, separation of whole sperm has
also not been possible for these animals (see Pinkel
et al., 1982). For example, investigators have tried
to separate sperm based on their differing DNA content
by density gradient sedimentation, but enrichment

15   results could not be verified -- one report claims
to have slightly enriched a fraction of bull sperm,
but not rabbit sperm (Schilling, 1971; Brandriff
et al., 1986). Attempted separation on the basis of
surface charge density imparted by DNA differences

20   has also been inconsistent (Hafs and Boyd, 1971), or
was based upon controversial quinacrine staining
(Garner, 1984).

           Another reason for these failures may be
that the head, tail, and plasma membranes of the

25   sperm, its other cellular material, and its highly
compact nucleus all act to mask the small DNA content
differences between X-sperm and Y-sperm. Some
evidence for this masking effect is the fact that
cytometric separation, while not feasible for whole

30   sperm, has been useful to prepare enriched subpopula-
tions of denuded sperm nuclei. Using this technique,
the sperm nuclei are first separated from the
membranes and other material of whole sperm. They
are then stained and partially sorted using a flow

35   cytometer (Johnson and Pinkel, 1986). The result
has been nuclei subpopulations enriched for the
X- and Y-chromosome.

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Investigators have also used this cytometric technique to test the results of various attempts to separate the X- and Y-sperm of whole sperm (a non-enriched sperm population). The Lawrence Livermore
5 National Laboratory and Oklahoma State University made a comparative study of several of the above-described "enrichment" approaches (Pinkel et al., 1985). They analyzed sperm separated by convection-counterstreaming-galvanizaton, albumin gradient,
10 density gradient, electromotility, and anti-H-Y antibodies. The results: "In no case was enrich-ment of either sperm population observed." (Id. at p. 130.) This finding is consistent with other studies of attempted enrichment: albumin density
15 gradient (Brandriff et al., 1986) and monoclonal anti-H-Y antibodies (Hoppe and Koo, 1984).

Monoclonal antibodies to sperm surface antigens which have heretofore been prepared also do not distinguish X- and Y-sperm. They bind to
20 both X-sperm and Y-sperm, and either inactivate or immobilize both types of sperm cells (Schmell et al., 1982, and Peterson et al., 1981). Monoclonal anti-bodies appear to inhibit sperm-egg binding without regard to whether they bind to the sperm acrosome,
25 head, midpiece, or tail. Further, antibodies specifically binding to the midpiece or tail have also been observed to immobilize sperm cells. (For a review of antibodies inhibiting fertility see Alexander and Anderson, 1987.)
30 .   Prior to this invention, it was not known whether one could isolate subpopulations of cells enriched in either X-sperm or Y-sperm. Nor was it known whether the plasma membranes of these enriched subpopulations would contain unique, sex selective
35 constituents, such as proteins, glycoproteins, or lipoproteins.

In light of these failures, we decided to
focus on the sperm cell surface as a possible tool
for sperm separation. Studies of the cell membrane
of unsexed mammalian sperm indicated that more than

5   1000 proteins are present on it. See, e.g., Noland
et al. (1983 and 1984); (Russell et al., 1983);
(Bradley et al., 1981); and (Hughes and August, 1981,
and Crichton and Cohen, 1983). All of these studies
used mixed membranes of both X- and Y-sperm. There-

10   fore, they failed to distinguish between membranes
and constituents characteristic of X-sperm-enriched
subpopulations and those of Y-sperm-enriched sub-
populations. Consequently, no one, until now, has
been able to perform an analysis of X-sperm or

15   Y-sperm membranes, to obtain usable quantities of
whole cells enriched for X- or Y-sperm, to identify
a sex-chromosome associated membrane protein of
mammalian sperm, or to isolate such proteins.

SUMMARY OF INVENTION

20   It is an object of this invention to provide
methods to increase the probability that mammalian
offspring will be of a desired sex or carry a gene
for a particular sex-chromosome linked trait. This
invention achieves this object by providing sex

25   associated membrane (SAM) proteins which are useful
as vaccines and to produce antibodies, themselves
useful as contraceptives or for providing semen
samples enriched in X- or Y-sperm.

       The present invention also provides for a

30   method to separate living cells based on DNA content.
The method involves separating the cells by flow
cytometry, the cytometer having been improved to
substantially increase its ability to recognize
fine distinctions in fluorescence. Sperm cell

35   populations separated in this manner result in
subpopulations enriched in X- and Y-sperm. By

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isolating the membranes from these enriched subpopu-
lations, one obtains another aspect of this invention:
X- and Y-enriched sperm-plasma-membrane vesicles.

This invention also provides for refined
5   and substantially pure sex-associated membrane (SAM)
proteins. These proteins are characterized in that
when one separates membrane proteins from enriched
sperm-plasma-membrane vesicles, SAM proteins are
more intense in one profile than the other. There-
10   fore, SAM proteins distinguish X- and Y-sperm from
each other.

One may use SAM proteins of this invention
to immunize females against X-sperm, Y-sperm, or
both, thereby increasing the probability of off-
15   spring of a certain sex, or decreasing fertility
altogether.

SAM proteins are also useful in preparing
another aspect of this invention: monoclonal and
polyclonal antibodies that selectively bind to X-
20   and Y-SAM proteins and, therefore, to X- or Y-sperm.
One may use these antibodies to produce semen
samples enriched for Y- or X-sperm. Upon incubation
with antibodies against X- or Y-SAM proteins, the
antibodies bind to and inactivate X- or Y-sperm
25   respectively, and prevent them from fertilizing an
ovum (Alexander and Anderson, 1987). The sperm
cells which have not been bound by the antibodies
are left viable and active for fertilizing ova.
Therefore, this invention provides for the first
30   time a method to produce a semen sample enriched in
active X- or Y-sperm capable of increasing the
probability that offspring will be of a desired
sex or carry a gene for a sex-chromosome linked
trait.

35   One may also use SAM proteins to detect
the presence of anti-SAM antibodies a sample.

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The antibodies of this invention are especially useful in methods of artificial insemination. Carrying out this embodiment of the invention, a viable semen sample usable for artificial insemi-
5   nation is mixed either _in vitro_ or _in vivo_ with a sex-selective antibody preparation of this invention. The resulting sample is enriched for X- or Y-sperm. One then uses the sample in the normal way in artificial insemination.

10   One may use the antibodies of this invention to separate semen into novel X- and Y-containing subpopulations, e.g., by affinity chromotography. These purified subpopulations are then useful in fertilizing ova to produce offspring of the desired
15   sex.

One may also use the antibodies which bind to the SAM proteins of this invention as a contraceptive by contacting sperm with both anti-X- and anti-Y-sperm antibodies.

20   ## DETAILED DESCRIPTION OF THIS INVENTION

In order that one may understand the invention described herein more fully, we set forth the following detailed description.

In this specification we mean the term
25   "protein" to include glyco-, lipo-, and phosphoproteins, polypeptides, and peptides, as well as complexes of these molecules.

By the term "refined" we mean proteins as least as pure as obtained by the 1-D gels we
30   describe herein.

By the term "substantially pure" we mean proteins at least as pure as obtained by the 2-D gels we describe herein.

This invention provides for refined or
35   substantially pure sex-associated membrane ("SAM") proteins. SAM proteins are characterized by dif-

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ferential existence on the membranes of X-sperm and
Y-sperm, respectively. The SAM proteins of this
invention are characterized by a molecular weight
(MW) determined by SDS-polyacrylamide gel electro-
5   phoresis (PAGE) and isoelectric point (pI) deter-
mined on immobilized pH gradient gels (IPG). The
SAM proteins of this invention are initially identi-
fied by a process comprising the steps of:
        (a)   sorting mammalian sperm into subpopu-
10   lations enriched for X-sperm and Y-sperm;
        (b)   separating the plasma membranes from
those enriched subpopulations; and
        (c)   identifying the X- and Y-sex-associated
membrane proteins among the plasma membrane proteins
15   of those enriched subpopulations by comparing the
relative amounts of protein present in corresponding
spots on two dimensional IPG-SDS/PAGE the plasma
membrane proteins from whole sperm, the plasma
membrane proteins from the X-enriched sperm sub-
20   population, and the plasma membrane proteins from
the Y-enriched sperm subpopulation.
        More preferably, the SAM proteins of this
invention are initially identified by comparing the
relative amounts of protein in corresponding spots
25   on two dimensional IPG-SDS/PAGE profiles of the
plasma membrane proteins representing the X-enriched
sperm subpopulation and Y-enriched sperm subpopula-
tion. Such two dimensional gels are preferred because
they result in the isolation of substantially pure
30   SAM proteins. They separate proteins on the basis
of two characteristics -- molecular weight and pI.
One dimensional gels separate proteins on the basis
of a single characteristic -- molecular weight or
pI. However, one-dimensional gels, such as SDS/PAGE
35   profiles (which separate proteins by molecular weight
only) or IPG profiles (which separate proteins based
on pI), are also useful in initially identifying the

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SAM proteins of this invention. The protein bands
so identified on 1-D gels contain refined SAM protein
but may contain a few other proteins of the same
molecular weight. The protein of that band may be

5   used directly as an immunogen. It may also be
further purified using conventional protein
purification techniques.

In this less preferred embodiment of
SDS/PAGE selection, the X-SAM proteins of this in-

10   vention, thus, are characterized in that they exhibit
a higher band density in the plasma membrane proteins
prepared from X-enriched sperm subpopulations as
compared to the corresponding bands for the plasma
membrane proteins from whole sperm and the plasma

15   membrane proteins prepared from Y-enriched sperm
subpopulations. Similarly, prepared in this aspect
of the invention the Y-SAM proteins of this invention
are characterized in that they exhibit a higher band
density in the plasma membrane proteins prepared

20   from Y-enriched sperm subpopulations as compared to
the corresponding bands for plasma membrane proteins
from whole sperm and the plasma membrane proteins
prepared from X-enriched sperm subpopulations.
More preferably, the X-SAM and Y-SAM proteins of

25   this invention exhibit the above described higher
relative spot densities on two dimensional
IPG-SDS/PAGE profiles.

After the SAM proteins of this invention
are initially identified as described above, one may

30   isolate them in large quantities from the plasma
membrane proteins of whole sperm using the molecular
weight, pI, or other physical, chemical, or biological
characteristics of the initially identified SAM pro-
teins. Thus, an important aspect of this invention

35   is that the time-consuming process of sorting sperm
into enriched X- and Y-subpopulations need only be

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done for initial identification of the SAM proteins
of this invention.

One may use the SAM proteins isolated from
an non-enriched sperm population or from the enriched
5   sperm subpopulations, as described above, in a variety
of ways in accordance with this invention. For
example, one may use them to innoculate females,
immunizing them against X- or Y-sperm, or both.
Also, one may use them to raise antibodies, either
10   polyclonal or monoclonal, using well known conven-
tional techniques. Furthermore, one could use SAM
proteins to detect the presence of anti-SAM
antibodies in a sample. This could be useful in
the diagnosis of infertility.

15   The novel antibodies produced in these
methods selectively bind to proteins on the plasma
membranes of either X- or Y-sperm. As such they are
useful in modifying semen to preselect the sex of
the offspring produced by it. For example, one may
20   use the novel antibodies of this invention _in_ _vivo_
or _in_ _vitro_ to bind either X-sperm or Y-sperm and
thus to select the sex of mammalian offspring. The
antibodies are especially useful in artificial
insemination and _in_ _vitro_ fertilization. They are
25   also useful in purifying X- or Y-SAM proteins or
the X- or Y-sperm of whole sperm by, for example,
affinity chromatography.

This invention is applicable to a wide
variety of species. For example, it is applicable
30   to the commercially important mammalian species --
cattle, dogs, cats, horses, swine, and sheep. It
is also applicable to humans.

In each of these species there is a DNA
content difference of greater than 1% as between
35   X-sperm and Y-sperm. Thus, one may treat sperm from
each of these species as described in this invention.
In each case the cells may be sorted into X- and

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Y-sperm enriched subpopulations, the plasma membranes
of those enriched subpopulations identified and
isolated, and, most preferably, the respective SAM
proteins from the plasma membranes of those sub-
5   populations identified and isolated. One may then
employ these proteins to produce sex specific anti-
bodies for use in the sex-selective and sex predic-
tive methods and compositions of this invention.

The SAM proteins of this invention are
10   also useful to isolate DNA sequences which code on
expression for SAM proteins. For example, one deter-
mines a partial amino acid sequence for a SAM protein.
Then one synthesizes, as a probe, a DNA sequence
encoding that amino acid sequence. One then con-
15   structs cDNA library of mRNA from a cell producing SAM
proteins. Then one probes the cDNA library with the
DNA probe using methods well known to the art.
After isolating clones containing cDNA hybridizing
to the probe, one identifies those cDNA sequences
20   encoding SAM proteins. One would do this, for example,
by expressing the cDNA in a eukaryotic expression
system and identifying clones producing protein which
binds to anti-SAM protein antibodies.

In the particular embodiment of this inven-
25   tion, specifically exemplified herein, we used bull
semen. Bull semen has a DNA content difference
between X-sperm and Y-sperm of about 4%. We sorted
the sperm of this semen into subpopulations enriched
by greater than 68% in either X-sperm or Y-sperm.
30   We then prepared the plasma membranes of these
enriched subpopulations and identified the sex-
specific components therein. We describe these
results in Example II.

We have also used the methods of this in-
35   vention, as described more fully herein, to identify
the plasma membrane proteins of subpopulations of
sperm enriched in X-sperm or Y-sperm and the com-

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ponents thereof in another species, the chinchilla.
In that species the X-SAM proteins have molecular
weights on SDS-PAGE as follows: 33 kD; 39 kD, and
53 kD. The Y-SAM proteins have molecular weights on
5 SDS-PAGE: 17 kD; 31 kD; 36 kD; 41 kD; 42 kD, and
57 kD.

          To enable more easily the practice of the
present invention, the following detailed directions
and experimental results are set out.

10   EXAMPLE I - Sorting Sperm by Flow Cytometry

          To sort mammalian sperm by means of a flow
cytometer so as to obtain separate viable cell
fractions or subpopulations enriched for X- and
Y-sperm, we used equipment and methodology which
15 have not heretofore been known. We now describe an
example of one such method.

          Flow Cytometer Modifications and Adjustments

          To carry out the sperm sorting, we modified
a commercially available flow cytometer. Our partic-
20 ular modifications were made on the Epics Model
752 Flow Cytometer sold by the Epics Division of
Coulter Electronics, Hialeah, Florida. However,
one can make similar modifications to other flow
cytometers. Flow cytometers, in general, operate
25 as follows.

          The flow cytometer utilizes a laser to
interrogate a sample stream of suspended cells
contained within an outer sheath fluid. For DNA
analysis, these cells are bound with a dye which,
30 in response to ultraviolet laser excitation, emits
fluorescent light in proportion to the amount of
DNA. A photomultiplier tube arranged orthogonally to
both the laser beam and sample stream receives this
light. Depending on the amount of DNA detected, one
35 can then separate cells into one of three containers

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(X, Y, or waste) employing the flow cytometer sorting
capability. In this operation, an ultrasonic vibrator
breaks the sample stream into individual droplets,
each containing an individual cell. The droplets
5   are given a charge based on the DNA content of the
cell and are deflected into the appropriate container
by an electric field. This instrument is capable of
analyzing and sorting cells at rates up to 10,000
cells/second.
10   We isolated the Model 752 Flow Cytometer
from vibrations of greater than 7 Hz by placing the
instrument on a fixed rigid tubular frame table
equipped with double deflection shear/compression
mounts. We further improved flow cytometer resolu-
15   tion by continuously degassing the sheath fluid
during the sort. We accomplished this by passing
the pressurized sheath fluid through expanded teflon
tubing designed for HPLC degassing. We injected
cells suspended in Hoechst 33342 DNA stain into the
20   sheath fluid using minimal pressure to reduce the
core size of the sample stream. We used a sample
insertion tube whose tip was bilaterally beveled and
polished to a 20° angle. The tube creates a ribbon
shaped sample stream whose flat surface is normal to
25   the long axis of the laser beam (See Stovel et al.,
1978). In this geometry, the viable sperm will
orient themselves such that their flat faces remain
normal to the laser illumination, thereby improving
the resolution.
30   We also made various modifications and
adjustments to the electronics of the Model 752 Flow
Cytometer: Potentials across the PMT (photomultiplier
tube) were set through a resistive voltage divider
network of low noise metal film resistors. The
35   signal was current-to-voltage converted utilizing an
op amp with characteristics of ultra high speed,
wide power band-width, low noise and high linearity,

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with coaxial capabilities. We used a Fast Fourier
Transform (1024 channel) of the signal for the
purpose of designing low-pass (1 MHz) and high-pass
(100 KHz) filters that bracketed the frequency of
5   the pulses associated with the X- or Y-sperm.
Signals representing the DNA were then actively
filtered and amplified before 10-bit analog-to-
digital conversion (ADC). All filter and amplifier
resistors were low-noise metal films tested for
10   linearity across the frequency of use. We set
amplifier gains at values of 10 or 20 and set PMT
voltages to detect signals in the upper channels of
the ADC.

        We also made various optical modifications
15   and adjustments to the flow cytometer. We adjusted
the laser for the fundamental transverse mode (TEM_{oo})
utilizing test microspheres (Epics Grade I Fulbright
Beads, CV < 1) or a divergent beam. Once adjusted,
the laser was peaked and aligned in TEM_{oo} at the
20   power output to be used for sorting (300-400 mW).
We initialized optical alignment by first using a
thread to confirm that alignment targets were in
horizontal alignment with the pinhole leading to the
PMT. Confocal optics (Epics) and other light path
25   components were set with the laser serving as a
reference point. The fluorescence emission of
microspheres resulting from 351 nm laser excitation
was detected through a completely light sealed path
containing two 408 nm long pass filters, having a
30   signal-to-noise ratio of greater than 1000. We
tuned the coefficient of variation (CV) with micro-
spheres to less than 1%. As a result of these
modifications, we have been able to improve the
signal-to-noise ratio to 1000:1.
35   In the preferred embodiment of this inven-
tion we also replaced the laser following indica-
tions of low performance; used a 4 hour warm-up

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before calibration and sorting; operated the flow
cytometer in a 20°C temperature-stabilized room with
black walls and dim lights; and shielded air-flow
arising from the machine fan which flowed across the
5   flow chamber.

We were able to resolve X- and Y-sperm
peaks, and thereby to sort the two populations, with
the following improvements in place: (1) a signal to
noise ratio of at least 100:1; (2) a laser power to
10   each cell of at least 90 mW; (3) a PMT quantum
efficiency of at least 8%; and (4) cells adjusted
to the same orientation. However, we obtained best
results when the signal to noise ratio is at least
1000:1; the laser power to each cell is at least 160
15   mW; and the PMT quantum efficiency is at least 20%.

Preparation of Sperm

Immediately after collection we diluted
freshly ejaculated mammalian semen with isoosmotic
PBS to 15 ml, and slowly cooled it to 5°C. At this
20   temperature, we washed the sperm three times in
isoosmotic, pH 7.2, tris-methylaminomethane buffered
saline (TBS) saturated with phenylmethylsulfonyl-
fluoride (PMSF) and 10 μg/ml Hoechst No. 33342
bis benzimide dye. This solution stained cells
25   and simultaneously inhibited enzymatic break-
down. We centrifuged the cells at 483 x g for 20
minutes and resuspended them to remove seminal
plasma proteins. We then diluted the washed cells
in dye solution to a cell concentration of 20 x 10^6
30   cells/ml and allowed them to stain for a minimum of
2 hours. (See Arndt-Jovin et al., 1977.) The Hoechst
dye is available from Calbiochem-Behring Corp., San
Diego, California. We prepared sheath fluid in the
same manner as the solution used to wash sperm, except
35   that the dye was omitted. We degassed the fluid as

described above. The objective was to match the
refractive indices between sheath and sample fluid.

Sorting of Sperm

We employed the Model 752 Flow Cytometer,
5   modified and adjusted as described above, for
sorting sperm. We sorted the sperm based on total
DNA content as measured with the aid of the Hoechst
dye. X-sperm have more total DNA content than
Y-sperm. The mean peak fluorescence arising from X-
10   and Y-sperm from bull, boar, ram, and other large
mammals is typically separated by values greater
than 3%. The whole sperm as prepared in the solu-
tion of TBS/PMSF/stain was analyzed at rates up to
10,000 cells per second. The cytometer sorted these
15   cells into X-enriched, Y-enriched, or waste popula-
tions. The X-enriched and Y-enriched populations
collected at a rate between 100 and 500 cells/sec.
Coincident cells were rejected. The actual flow
rate used in any sort depends on the state of the
20   machine, with a reduction in flow rate required to
improve resolution. We adjusted the flow rate to
observe a plateau on the DNA histogram, indicating
the onset of splitting into X and Y peaks. We often
set the optimal sheath-to-sample flow at a differ-
25   ential pressure greater than pinch-off by 2 mm Hg.
We set sorting gates such that only the cells con-
tained in the outer third of the DNA histogram for
oriented X-chromosome bearing and Y-chromosome
bearing sperm were collected. The anti-coincidence
30   circuit was preferably active during sorting.

In this way we simultaneously collected
two viable subpopulations enriched for X- or Y-sperm,
respectively. Each subpopulation had an enrichment
of at least 68%. We were also able to obtain Y-sperm
35   subpopulations enriched to 72%. Such concentrations
were sufficient for the purpose of the present inven-

tion and were used, as will subsequently be described,
for isolation and identification of the novel, enriched
plasma membranes vesicles and the SAM proteins of
this invention. Each milliliter of sorted cells

5   contained approximately 300,000 cells and required
about 30 minutes to sort. Roughly 20 x 10^6 cells
could be collected per week.

        EXAMPLE II - Isolation of Sex-Specific
                     Sperm Plasma Membrane Vesicles

10   We used the X-sperm or Y-sperm enriched
cell subpopulations of Example I to isolate plasma
membrane vesicles (PMV), and X- and Y-enriched
non-membrane sperm component.
                We cavitated cell samples containing

15   enriched X-sperm or Y-sperm subpopulations in Parr
bombs. Suitable sample sizes were 3 to 10 ml with
50,000 to 500,000 cells/ml. We also cavitated non-
enriched sperm samples (50:50 mixture of X- and
Y-sperm). We used a cavitation method (at about

20   650 psi) as described by Gillis, et al., 1978. We
separated plasma membrane vesicles consisting of
mostly (e.g., 80% (boar sperm data)) head plasma
membrane and some (e.g., 20% (boar sperm data)) tail
plasma membrane from sperm heads, tails, and other

25   particulates by pelleting centrifugation twice at
2500 x g for 30 minutes. We withdrew the supernatant
containing the PMV material and centrifuged it at
100,000 x g to obtain the PMV material, which we
resuspended and washed in 10 mM tris acetate (pH

30   5.5). This removed most of the TBS/PMSF/stain from
the isolated PMV. Using this procedure we obtained
three plasma membrane vesicles populations:
(1) X-enriched sperm plasma membrane vesicles
(PMV-X) (approximately 68% X-sperm); (2) Y-enriched

35   plasma membrane vesicles (PMV-Y) (approximately 72%
Y-sperm); and (3) non-enriched plasma membrane

vesicles (PMV-X/Y) (approximately equal amounts of
X- and Y-sperm). The pelleted material resulting
after centrifugation of X- and Y-enriched sperm
subpopulations is the X- and Y-enriched non-membrane

5   sperm component.

   One may use enriched PMV and enriched non-
membrane sperm component to identify an array of sex-
associated molecules which exist predominately in X-
or Y-sperm. This includes X- and Y-SAM proteins

10   and other SAM molecules (such as lipids and carbo-
hydrates), and X- and Y-non-membrane sex-associated
molecules (such as cytoplasmic proteins or other
molecules). One identifies these molecules using
techniques analogous to the ones we describe for

15   identifying SAM proteins.

        EXAMPLE III - Identification of SAM
                    Proteins on 1-D Gels

   We used PMV-X and PMV-Y of Example II to
identify X-SAM and Y-SAM proteins on 1-D gels. We

20   began by solubilizing PMV-X and PMV-Y in 2% SDS and
^{125}I labelled using chloramine-T (Stanley et al.,
1971; Frost, 1977). We separated the proteins by
Laemmli SDS/PAGE (5%-15% T; 5% C). For comparison
we loaded three lanes on a single gel with PMV-X,

25   PMV-Y, and molecular weight standards, respectively.
We conducted the electrophoresis at 100 V constant
through the stacking gel/125 V constant through the
separation gel with water cooling.

        We compared autoradiographs (after 1 week)

30   of the X- and Y-profiles by observing a band on one
profile and comparing it with the corresponding
location on the other profiles, looking for an
increase or decrease in density. When the density
of a band in the X-profile was increased in com-

35   parison to the Y-profile, we designated that band
an X-SAM protein. If the band was increased in
density on the Y-profile and decreased in density on

the X-profile, then we designated it a Y-SAM protein.
As an internal control we also compared the density
of bands to the density of the corresponding bands
on the X/Y profile (prepared from PMV-X/Y.) This

5   band should be intermediate in density as compared
to the bands of the X- and Y-SAM proteins. We then
compared protein bands designated SAM on autoradio-
graphs with the molecular weight standards in order
to approximate the molecular weight of the identified

10 SAM proteins.

We ran similar comparative gels using
silver staining to identify the various proteins.
Again, by comparison among the X-, Y- and X/Y
profiles we identified the SAM proteins of this

15 invention. In comparing various gels of separated
proteins of PMV-X, PMV-Y and PMV-XY, the molecular
weight standards are important. The molecular
standards are aligned such that each standard
overlaps with the corresponding molecular weight

20 standard on the other gel. With this base for
comparison, one may obtain a visual identification
of the desired SAM proteins by location, color, and
staining density relative to other bands on a gel
profile.

25 Because of this ability to compare gels,
once the X- and Y-SAM proteins of this invention
are identified on a gel of membrane proteins from
enriched sperm sub-populations, one may use those
molecular weight locations to identify the X- and

30 Y-SAM proteins on gels of plasma membrane proteins
from whole (non-enriched) sperm.

An important attribute of this invention
is that cell sorting and component enrichment need
only be done once, to originally identify specific

35 SAM proteins. Subsequently, one may use the gel
characteristics of the SAM proteins themselves to

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identify and to isolate large amounts of those proteins for further study and use.

One may then isolate SAM proteins from the PMV-X and PMV-Y by one of the following methods. In
5   the first method, SDS/PAGE profiles (Laemmli, 1970) of PMV-X and PMV-Y are run side-by-side and silver stained (Wray et al., 1981). In the second method, solubilized X- and Y plasma- membrane proteins are iodinated with ^{125}I using chloramine-T (Stanley and
10   Haslam, 1971) and a similar SDS/PAGE profile is run and autoradiographs of the separated proteins are obtained. In either case we isolated the SAM proteins as described in Example IV.

EXAMPLE IV - Bull SAM Proteins
15   Identified by 1-D Gels

We identified bands in 1-D gels which con- tain SAM proteins from bull sperm. We processed bull sperm as previously described, separated the proteins from X- and Y-enriched sperm plasma membrane
20   vesicles by SDS/PAGE, and determined the molecular weights of bands of increased density on one as opposed to the other. We found bands containing X-SAMs of the following molecular weights: 19 kD, 25 kD, 29 kD, 32 kD, 39 kD, 72 kD, and 120 kD. We
25   found bands containing Y-SAMs of the following molecular weights: 15 kD, 45 kD, 57 kD, 64 kD, and 125 kD.

Although this method accurately identifies bands containing SAM proteins, and one can isolate
30   refined SAM proteins by cutting-out the bands on these gels, this is not the preferred method for precisely measuring the molecular weights of SAM proteins. This system has a MW standard deviation of 4.2% and some bands may contain more than one SAM
35   protein of slightly different molecular weight.

Nevertheless, for the purposes of the present invention, it is not essential to determine

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whether the SAM bands identified only by molecular
weight (Example III) contain only one protein.
Without making any further separation or charac-
terization of the proteins of a given band, one can
5   use the bands, which contain refined SAM proteins,
directly to prepare antibodies (either polyclonal or
monoclonal) which will bind selectively to X- or
Y-sperm. One can employ these antibodies in the
methods and compositions of this invention for
10   increasing the probability that offspring will be
of a desired sex. The procedures which are used
are described below.

EXAMPLE V - Identification Of SAM
Proteins By 2-D Gels

15   Although the SDS/PAGE gels described in
Example III permit the identification of the SAM
proteins of this invention and a characterization of
those proteins by molecular weight, it is preferable
to use two dimensional IPG-SDS/PAGE gels to identify
20   and characterize SAM proteins and to isolate substan-
tially pure SAM proteins. Two-dimensional gel
analysis is a process in which proteins are separated
first in one dimension by their net electrical charge
(pI) and next in a second dimension by their mole-
25   cular weight. We performed 2-D gel electrophoresis
of plasma membrane proteins from X- and Y-enriched
sperm subpopulations and from non-enriched sperm
produced X-, Y- and X/Y profiles, respectively.
We purchased equipment for horizontal
30   isoelectric focusing, including casting molds,
rehydration cassettes, Immobilines® (acrylamide
derivatives) pK 3.6, 4.6, 6.2, 7.0, 8.5, 9.3, LKB
ampholine (carrier ampholyte) 3.5-10, and gel bond
PAG film from LKB. We purchased additional carrier
35   ampholyte (3-10) from Pharmacia and Serva. We pur-
chased acrylamide from Amresco; bis from FMC; SDS

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from BDH; urea from Schwarz/Mann. We purchased all
other chemicals from Sigma.

In a preferred 2-D gel separation process
of this invention, we began with solubilized plasma
5   membrane vesicles from whole sperm and from X- and
Y-enriched sperm subpopulations isolated as in
Example II. The LKB 2117 Multiphor II Electro-
phoresis System laboratory manual gives instructions
and formulations for pouring 5.0% T, 2.7% C, 0.5mm
10   thick IPG gels with a broad range pH gradient of
4-10. We allowed gel polymerization to proceed for
1 hour in an oven heated to 50°C. Following poly-
merization, we removed gels from the mold, washed
them twice for 30 minutes in HPLC water and then
15   rinsed them for 30 minutes in a solution of 2.5%
glycerol. We air-dried the gels overnight in a dust
free cabinet. Prior to focusing, we rehydrated IPG
gels with a solution consisting of 8 M urea, 10 mM
dithiothreitol (DTT), 0.5% (volume/volume) nonidet
20   p-40 (NP-40), 0.5% carrier ampholyte (CA). We
utilized different brands of carrier ampholyte (LKB
ampholine®, Pharmalyte®, and Servalyte® 3-10) to
insure the best possible pH distribution. It is
important that the carrier ampholyte used span the
25   entire pI range over which the sample is to be
focused. We included carrier ampholyte in rehydra-
tion of the gel in order to decrease hydrophobic
interaction with basic immobilines.

We solubilized bull plasma membrane pro-
30   teins with a solution containing 9 M urea, 2%
(weight/volume) DTT, 2% (volume/volume) NP-40,
0.8% (volume/volume) carrier ampholyte. To aid in
solubilization, we sonicated samples in a water
bath sonicator at 4°C for 10 minutes. We pelleted
35   remaining aggregates by centrifugation at 13,000 x g
for 10 minutes.

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We loaded the solubilized samples in
preformed wells near the anode. Anolyte and
catholyte were 10 mM phosphoric acid and 10 mM
sodium hydroxide respectively. Focusing parameters
5   were 170 volts, 2 mA, and 5 W until 1000 volt hours,
followed by gel dependent increases to 1700 volts
for a total of 7000 volt hours. Lower starting
voltage improved protein entry. The pH gradient was
measured in 1 cm increments with a calibrated LKB pH
10   surface electrode immediately following run.

Following isoelectric focusing, we cut
the gel into strips corresponding with sample wells.
We incubated the strips in equilibration buffer
and loaded them directly onto SDS slab gels. We
15   equilibrated for 30 minutes with gentle shaking at
room temperature in 8 ml of a solution containing
0.05 M tris-HCl pH 6.8, 6 M urea, 2% (weight/volume)
SDS, 1% (weight/volume) DTT, 30% glycerol and 0.001%
bromphenol blue. Following equilibration we rinsed
20   the gel strips briefly to remove excess equilibration
buffer and loaded them directly onto vertical
11.0% T, 2.7% C, 1.5 mm thick SDS slab gels with
a stacker of 4.8% T, 2.7% C. We electrophoresed
proteins at 100 volts until the dye front moved
25   through the stacker. We then increased the voltage
to 140 volts for the remainder of the run.

Upon staining, these 2-D gels produced
hundreds of spots. Using internal standards, we
determined the molecular weight and pI of these spots.
30   We measured the integrated intensity of the gel spots
using a BioImage scanner (Kodak). We determined the
mean integrated intensity of the spots from about
thirty X/Y profiles and from about three X- and three
Y-profiles. Then we calculated for each protein
35   spot the difference in the mean integrated intensity
on the X-profiles and the mean integrated intensity
on the Y-profiles. We designated a protein as a SAM

protein when this difference was at least 1.8 standard
deviations from the mean integrated intensity of the
protein on the X/Y profiles. X-SAM proteins were,
of course, more intense on X-profiles, and Y-SAM

5   proteins, on Y-profiles. The SAM proteins we identi-
fied are presented in Table 1. The molecular weights
are accurate to within 4.2% and the isoelectric
points, to within +/- 0.16 pH. We do not intend
this list to exhaust the entire class of SAM pro-

10 teins -- more, undoubtedly, exist. For example, our
examination of the 2-D gels revealed many spots which
appeared on one profile but not on the other. In
some cases, the absence of a spot indicates a protein
whose intensity is below the range of sensitivity

15 for detection. Clearly, one of ordinary skill in
the art could visualize these proteins by loading
more sample on the gel or by using more sensitive
staining techniques. Then, using the techniques
advanced in this invention, one could identify

20 which among these are SAM proteins.

TABLE I

	X-SAM		Y-SAM	
	MW(kD)	pI	MW(kD)	pI
	20.9	5.74	9.6	6.52
25	26.3	7.58	19.9	5.67
	27.8	6.08	29.0	6.67
	44.1	6.90	30.3	5.77
	52.5	5.33	36.5	7.16
	58.0	5.99	41.1	6.21
30	59.4	6.59	55.5	6.82
	59.5	6.81	55.9	5.25
	62.1	7.23	58.0	8.67
	62.5	5.54	62.9	6.34
	62.7	6.85		
35	62.8	6.64		
	63.9	5.83		
	68.2	5.95		
	78.6	7.14		

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EXAMPLE VI - The H-Y Antigen Is not a SAM Protein

To determine whether or not the H-Y antigen, previously asserted to be sex associated, is a
5   SAM protein, we isolated whole sperm plasma-membrane proteins and separated them as described on 2-D gels. Then we performed an immunoblot as described in Example IX using anti-H-Y monoclonal antibodies which Dr. G.C. Koo generously supplied to us. The
10   blot revealed eight proteins having an epitope recognized by anti-H-Y monoclonal antibody. We determined the molecular weights and pI for these proteins and present them in Table II. Significantly, none of these proteins matches any of those
15   we identified as SAM proteins in Table I. Therefore, the H-Y antigen is not a sex-associated membrane protein as defined in this invention.

TABLE II

H-Y Antigen*

MW(kD)	pI
23.4	6.96
39.5	5.89
34.8	5.96
42.6	6.80
38.4	6.96
41.6	7.58
57.5	6.00
56.1	5.91

EXAMPLE VII - Use Of Gel Profiles

30   Larger amounts of the SAM proteins of this invention one are obtained by isolating the proteins from whole sperm.

_____

*   Bull sperm plasma-membrane proteins separated by
35   2-D gel which bound anti-H-Y monoclonal antibody in immunoblot.

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In one embodiment of that process, we
prepared one-dimensional SDS/PAGE gels of PMV-X,
PMV-Y, and PMV-XY as described in Example III.
(Alternatively, a two-dimensional gel as described
5   in Example IV is used.) We then transferred the
protein bands from the PMV-X/Y gel to a set of
sheets of nitrocellulose (NC) by transblotting. In
this technique, one positions the gels and NC sheets
adjacent to one another, and applies a constant
10   electric current to transfer all of the protein
bands on the gels to the NC sheets, maintaining
their relative positions. In one embodiment of our
invention, we performed the transfer using SDS/PAGE
1D gels in the presence of 25mM Tris, 192mM glycine,
15   and 20% methanol (Towbin et al., 1979) using 250mA
of constant current for about 16 hours at 4°C.

Following the transfer to NC, we stained
the bands with 0.5% amido black in 7% acetic acid.
(The technique of using amido black is described by
20   Schaffner et al. (1973), and this stain is available
commercially from Sigma Chemical Co., St. Louis,
Missouri.) We prefer to use amido black stain
because it does not interfere with the subsequent
preparation of anti-sera or hybridomas from proteins.
25   In the embodiment of this invention using
the 1D SDS/PAGE gels, to identify the SAM protein
bands on the NC sheets for use in immunizing animals,
we aligned silver stained profiles of the SDS/PAGE
gel of molecular weight separated SAM proteins as
30   described in Example III (with molecular weight
standards) with the molecular weight standards on
the amido black stained transblotted SDS/PAGE 1D
profiles. The alignment of the molecular weight
standards allows the matching of the silver stained
35   SAM bands on the SDS/PAGE gel with the corresponding
amido black stained protein bands on the NC sheets.
We then cut out the amido black stained bands on the

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NC sheets that correspond to SAM proteins using a
razor blade.

        We used the cutout bands to isolate the
desired SAM proteins or to prepare directly anti-
5   bodies to these proteins. For example, in one
embodiment the cutout bands are surgically
implanted to raise antibodies. Alternatively,
the proteins in those bands are extracted with a
suitable solvent, such as dimethylsulfoxide (DMSO)
10   and injected the into a test animal. We prefer the
latter procedure. In such an extraction, we prefer
to extract the NC band with 100 µl DMSO. We then
mixed the resulting DMSO solution with 1 ml of
adjuvant (Freund's complete) prior to injection to
15   raise antibodies. (The preferred embodiment is
100 µl of adjuvant.)

        There is now available to the art means
to isolate the binding portion of the immunoglobulin
molecule. One example is a kit to isolate Fab or
20   F(ab')$_2$ fragments, available from Pierce Corporation,
Rockford, Illinois. When we refer to antibodies in
this specification, we therefore mean to include
fragments such as those which contain only the
binding portion.

25   <u>EXAMPLE VIII</u> - Hybridomas And
                 <u>Monoclonal Antibodies</u>

        We prepared several hundred hybridomas
using 150 pooled NC strips, prepared as described
above, which bracketed the 19,000 and 25,000 dalton
30   X-SAM bands and the 57,000 and 64,000 dalton
Y-SAM bands, respectively (+/- 5%) (bull sperm).

        For hybridoma production, we used a non-
secreting Balb/c mouse line SP2/0-AG14 obtained from
the American Type Culture Collection, Rockville,
35   Maryland. We used ATCC CAT1581-CRL (batch F-5286)
as our myeloma line. We grew cells in 10% FCS/DMEM

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or RPMI-1640 (we prefer the latter) and maintained
between 0.2-1.0 x 10^6 cells/ml.

5   We immunized mice using the SAM/DMSO/
adjuvant mixture described in Example VII. Between
20 μg and 200 μg of protein was used per mouse. Two
weeks later and four days prior to the collection of
spleens and fusion, we injected subcutaneously a
boost of 100-150 μg of PMV-X/Y or 1 x 10^7 whole sperm
in PBS to stimulate X or Y specific clones that pro-
10   duce antibodies to the native SAM protein configura-
tion. (We prefer IP injection of whole sperm.) It
should, of course, be understood that one may use
other protocols to immunize the mice. For example,
the mice could be treated as above with the SAM/DMSO/
15   Freund's complete adjuvant mixture, described above,
boosted once per day for 4 days with PMV-XY in PBS,
and the spleens collected.

One day prior to fusion, myeloma cells
were split to ensure that they were in log phase
20   growth. On the day of fusion, the animal was
sacrificed and splenectomized. We rinsed the spleen
in sterile DMEM or RPMI-1640 (we prefer the latter)
and teased it to separate splenocytes. (We now
separate using syringe and needle perfusion.)
25   Typical cell recovery from 1 spleen was 1 x 10^8
cells. We washed splenocytes 3 times in DMEM or
RPMI 1640. We prefer the latter.) The myeloma
cells were also washed 3 times. We counted
splenocytes and myeloma cells and mixed them
30   together at a ratio of 7 spleen cells:1 myeloma
cell. (We currently use 1:1.) We then centri-
fuged the cells at 1000 rpm (Mistral 3000) and
decanted the supernatant.

Fusion was done with 1 ml of PEG for a
35   period of one minute with gentle agitation. (We
currently adjust PEG to pH 7.) We stopped the
reaction with 20 ml DMEM (or preferably RPMI 1640)

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and centrifuged the mixture as before. We decanted
the DMEM (or preferably RPMI 1640) and gently
resuspended the pellet of fused cells in 12 ml of
HAT (hypoxanthine aminopterin thymidine) medium and
5   plated the cells into twenty-four well plates (1 ml
of cell suspension/well). (We currently use HMT --
hypoxanthine methotrexate thymidine.) We incubated
plates overnight in a 7% CO_2 incubator at 37°C. The
next day we fed the wells an additional ml of HAT
10   (or HMT) medium and left them to incubate for 7 to
14 days. We removed the HAT (HMT) medium and replaced
it with 10% FCS/DMEM or RPMI. (We prefer the latter.)
We incubated plates until colony formation was
visible. After the colonies had expanded into larger
15   cultures, we froze them at -70°C and stored them
long-term in liquid nitrogen.

(1)   ELISA Assay

We screened supernatants by an ELISA assay
as follows: 96-well microplates were coated with
20   100 µl of PMV-X/Y (2 µg/ml) in 0.05 M carbonate-
bicarbonate buffer (pH 9.6) for 16-18 h at 4°C in
a humid chamber (or for 2-4 h in a 37°C incubator).
The carbonate-antigen solution may be used 2-3 times
for blocking plates (which may then be stored for
25   subsequent use at -20°C). We incubated the plates
for 30 min at room temperature, added 1×10^5 sperm
cells per well and incubated the wells at 4°C
overnight in the humid chamber. (We prefer whole
cells as antigens.)
30   We washed the plates with 200 µl of 0.05%
Tween 20 in 0.02 M phosphate saline buffer (pH 7.2)
three times at room temp. The wells may be filled
with 200 µl of 3.0% gelatin in PBS-Tween, incubated
for 30 min at room temperature in a humid chamber,
35   and the blocking solution (gelatin-PBS) aspirated-off.
However, preferably we omitted these steps and instead

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immediately incubated the plates with 100 µl of normal
(from either a non-immunized mouse or media as above --
a negative control) and immune supernatants (undiluted)
at 37° for 1 hour, in a humid chamber, and then wash
5   the plates as above. Next, 100 µl of peroxidase
conjugated goat anti-mouse IgG in the appropriate
dilution in 0.5% PBS/tween 20 was added to each
well and incubated for 1 h at 37° in a humid chamber
and the plates washed as before. We added 100 µl of
10   a 1:2 mixture of TMB(3,3',5,5' tetramethylbenzadine)/
H_2O_2 to each well and incubated the plates for 60 min
at room temperature. We determined color development
visually or determined absorbance at 660 nm using
a Beckman Biomek 1000 robotic arm.

15   In these assays, we have assumed that the
ratio of the absorbance reading of a highly positive
sample to that of a negative sample in indirect ELISA
should be at least 5:1. We also considered as posi-
tives all wells with results greater than 3 standard
20   deviations from the mean of the negative controls.

             We cloned positive cell lines by limiting
dilutions of 1 cell/well and 3 cells/well. We
expanded each well by moving the contents of the
well to a larger well and finally to a flask. We
25   stopped such expansion at a 150 cm^2 sized flask.

             EXAMPLE IX - Immunoblots Of SAM
                         Proteins And Monoclonal
                         Antibodies

             We analyzed monoclonal antibodies produced
30   from those hybridomas that were positive in the
ELISA assay produced above for binding activity
against the X-SAM and Y-SAM proteins of this inven-
tion.
             We washed the nitrocellulose sheets carrying
35   the SDS/PAGE protein bands, described above, three
times (5 min/wash) in a washing solution (20 mM

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Tris-HCl (pH 8.2), 20 mM sodium azide, 0.9% NaCl and
0.1% BSA). (We now prefer 0.8% NaCl, 0.02% N_2PO_4,
0.144% $Na_2PO_4 \cdot H_2O$, 10 mM sodium azide, and 0.1% BSA).
They were then blocked by incubation with the washing

5   solution augmented with 4.9% BSA to a final BSA con-
centration of 5% and shaking for 45 min. Alterna-
tively, we blocked overnight at 4°C. After washing
3 times as before, we placed the NC blots in a mini-
blotter. We then applied the monoclonal antibodies

10   (mAbs) to the nitrocellulose blots. For a 25 µg
transblot, we diluted mAb supernatants 10 times with
the washing solution, augmented with 1% normal or
total goat serum. We pipetted 130 µl of the diluted
mAb supernatants in duplicate into the apparatus.

15   As a negative control we used diluted washing
solution lanes in duplicate. As a positive control
we used antibodies that bound to greater than 90%
of all sperm. We incubated the blots with the mAbs
or the positive and negative controls for 30 minutes

20   and then washed them several times to ensure removal
of all unbound antibody.

    Following washing, we added 130 µl Auro-
probe BL+ stain (Janseen) to all of the lanes and
incubated them for at least 2.5 h (incubation for

25   5 h is also permissible). We washed the lanes
several times (3 min/wash) with washing solution,
removing the liquid with the vacuum manifold.

    We placed the blot in a clean dish, washed
again with distilled water and incubated the blot

30   with Intense II silver stain (Janseen) (about 70 ml)
for 10 min. We rinsed 3 times with distilled water
(5min/rinse). (We now prefer 15-40 min rinse.)
Results were positive when color density was greater
than negative control.

35   We found that these monoclonals bound
to 1-D gels at molecular weight regions corres-
ponding to SAM proteins identified in Example IV.

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EXAMPLE X - Modification of Semen

        Polyclonal or monoclonal antibodies of this
invention are useful to modify mammalian semen to
increase the percentage of male or female offspring

5   resulting from fertilization by the modified semen.
Antibodies binding to X- or Y-SAM proteins of this
invention, as described above, when incubated with a
semen sample, bind to X- and Y-sperm, respectively.
Sperm that are bound with antibodies become

10 inactivated -- their mobility is impeded and they
become ineffective at fertilizing an ovum. For
assured effectiveness, the antibody preparation is
preferably produced from the sperm of the same
mammalian species with which it is to be used.

15 However, one can expect sex-selective cross-
reactions to occur for sperm of other animals. Con-
sequently, our antibody preparations are useful for
modifying sperm of more than one species of animal.
        It will be clear that in any of the methods

20 we describe immunogenic fragments of the SAM proteins
or other proteins which induce antibodies cross-
reactive with SAM proteins are useful for immuni-
zation.
        We added an antibody that had been raised

25 to a SAM protein (100-500 ug per million sperm) to
neat semen. The semen was either collected and
frozen in liquid nitrogen, or it may be freshly
ejaculated. The antibodies were preferably in a
physiologically acceptable carrier formulated to

30 have the properties of maintaining the activity of
the antibodies, being non-toxic to sperm, and con-
ducive to binding of the antibodies to the sperm.
For example, we employed a sterile, buffered aqueous
carrier which had a physiologically acceptable pH

35 and salt content. The carrier had a pH from 6.4 to
8.4 and contain a phosphate buffer. A unit dose
form of such a preparation should contain sufficient

antibodies, for example, to preferentially deacti-
vate all of the X-bearing or Y-bearing sperm in an
artificial insemination dose of semen. Antibodies
are best stored lyophilized.

5   Preferably, the antibodies are incubated
with the semen sample for 15-60 min at 37°C.
Following incubation, the mixture is used directly
for artificial insemination (AI) or is frozen in
liquid nitrogen according to standard procedures for
10   later use. The antibodies can be added at any time
during the course of processing a semen sample. The
important steps are: (1) the antibody is given ade-
quate time to bind to sperm; and (2) the antibody
to sperm ratio is set such that there is excess anti-
15   body. Using either monoclonal or polyclonal anti-
bodies, the important factors are incubation time
and antibody to sperm ratio. In one embodiment the
incubation is carried out _in vivo_, such as by simul-
taneously introducing the semen and the antibody
20   preparation (preferably premixed with the semen)
into the vagina of the female mammal. Alternatively,
the antibodies are added to the petri dish during
in vitro fertilization.

          In a preferred method of artificial insemi-
25   nation, monoclonal antibodies are mixed with freshly
ejaculated semen in such proportions to give excess
monoclonal antibody (i.e., unbound to sperm). The
combination of fresh semen with monoclonal antibody
is then mixed with cryopreservatives and packaged
30   in straws in the then usual fashion (industrial
standards). The straws are used in the usual methods
for artifically inseminating cattle.

          EXAMPLE XI - Monoclonal Antibody Binding
                      To Whole Sperm

35   We bound monoclonal antibodies to whole
sperm by the following preferred method: We thawed

antibody supernatants in a 37°C bath and diluted
with a combination of Dulbecco's/PBS, Hoechst stain,
and BSA as previously described. We diluted a whole
sperm sample with a labeling solution to a final
5   concentration of 480 x 10^6 sperm cells per 12 mls.
We added monoclonal antibody (ca. 200 µl supernatant)
to 1 x 10^6 cells and allowed it to incubate for 1 hour
at room temperature. We centrifuged the cells at
100 x g for 5 minutes, discarded the supernatant and
10   resuspended the cell pellet in 200 µl of a 1:20 dilu-
tion of an affinity-purified anti-mouse antibody
conjugated with R-Phycoerythrin. Following a second
centrifugation, we discarded the supernatant and
resuspended the cell mixture in a 10% neutral buffered
15   formalin solution.

        EXAMPLE XII - Other Uses of SAM Proteins

              SAM proteins are also useful in vivo to
influence the sex of the offspring. In one
illustrative method, a female mammal is immunized
20   with a SAM protein preparation. The immunization is
carried out so that it is effective to generate
polyclonal antibodies, which will be present in the
reproductive fluid of the female mammal for selective
binding to X-SAM or Y-SAM when semen is introduced
25   into the vagina. Immunization is as previously
described with the site of immunization being that
which produces antibodies that are sex specific.
The animal is then allowed to mate naturally.
              The proteins are also useful to decrease
30   fertility by immunizing a female as just described
with both X- and Y-SAM proteins.
              The SAM proteins of this invention are
also useful to detect the presence of anti-SAM
antibodies in a sample. The presence of antibodies
35   in a serum sample might explain infertility or the

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unusual proclivity to produce offspring of a certain
sex. One might test a sample by performing an ELISA
assay such as in Example VIII using SAM proteins
rather than PMV as the substrate.

5   EXAMPLE XIII - Other Uses of Antibody
                        Preparations

         The antibody preparations of this invention
are useful for other purposes besides modifications
of semen to increase the percentage of male or female
10   offspring. For example, the sex specific antibodies
of this invention are useful in an affinity column
to extract quantities of SAM proteins from a mixture
of substances. If the substances and SAM protein
are contained in a lipid microdomain then those sub-
15   stances that are associated with that protein can
also be enriched. In the embryo transplant industry,
those SAM proteins that migrate or are found in the
fertilized egg may be identified using labeled
antibodies that have been raised to SAM proteins,
20   thereby sexing the embryo. Should those SAM anti-
gens, associated with the fertilized egg, escape
into the maternal system, incubating maternal body
fluids with labeled anti-SAM protein antibodies, or
using SAM proteins or determinants thereof, will
25   allow for prenatal sex and pregnancy determination.
         Antibody preparations of this invention
can also be used for identifying plasma membrane
proteins associated with either the X-chromosome or
Y-chromosome. In still another use, the antibodies
30   are useful for identifying non-sperm polypeptides
or proteins which exhibit antigenic properties
capable of generating antibodies which bind selec-
tively to Y-sperm or X-sperm. In a related use, the
antibodies are useful for isolating those polypep-
35   tides or proteins. Another related use is that of
utilizing antibodies raised to Y-SAM proteins or to

X-SAM proteins for isolating polypeptides or pro-
teins that are associated in the microdomain of the
proteins that bind to these antibody preparations.

EXAMPLE XIV - Sex-Chromosome Linked Traits

5   The SAM proteins of this invention are
also useful for increasing or decreasing the
probability that offspring will carry a gene for a
particular sex-chromosome linked trait. Sex-
chromosome linked traits are genetic characteristics
10   determined or controlled by genes on either the X-
or Y-chromosome that therefore show a different
pattern of inheritance in males and females. For
example, in humans, color blindness and hemophilia
are X-chromosome linked traits.
15   We have already described how to use SAM
proteins to produce antibodies which bind to sperm
carrying a particular sex chromosome. Because these
antibodies inactivate sperm according to the sex
chromosome they carry, they are also useful for
20   inactivating sperm carrying a gene for a particular
sex-chromosome linked trait. For example, if one
wished to decrease the probability that offspring
carry a gene for a particular X-chromosome linked
trait, one would incubate the semen sample with anti-
25   bodies binding to X-SAM proteins. This would inac-
tivate the X-sperm carrying the undesirable gene.
Conversely, if one wished to increase the probability
that offspring carried a particular gene for a
X-chromosome linked trait, one would incubate a semen
30   sample with antibodies binding to Y-SAM proteins.
This would inactivate Y-sperm, leaving viable the
X-sperm which carried the desirable gene. Likewise,
the corresponding procedures are used for genes for
Y-chromosome linked traits.

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While we have hereinbefore described a
number of embodiments of this invention, it is
apparent that one of skill in the art could alter
our procedures to provide other embodiments which
5   utilize the processes and compositions of this
invention.

Therefore one will appreciate that the
scope of this invention is to be defined by the
claims appended hereto rather than the specific
10   embodiments which we have presented by way of
example.

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CLAIMS

1.   A method of sorting living sperm cells whose DNA content difference is greater than 1% comprising the steps of:

(1)   staining living sperm cells with a dye which, in response to laser excitation, emits fluorescent light in proportion to the amount of DNA in the cell; and

(2)   sorting the sperm cells according to their fluorescence with a flow cytometer into subpopulations enriched in X-chromosome-bearing sperm cells and Y-chromosome-bearing sperm cells, said flow cytometer

(a)   having a signal to noise ratio of at least 100:1,

(b)   delivering a laser power to each cell of at least 90 mW,

(c)   having a PMT quantum efficiency of at least 8%, and

(d)   being capable of adjusting the sperm cells into the same orientation.

2.   The method according to claim 1, wherein greater the signal to noise ratio is at least 1000:1, the laser power delivered to each cell is at least 160 mW, and the PMT quantum efficiency is at least 20%.

3.   A viable subpopulation enriched for X-sperm comprising at least 68% X-sperm.

4.   A viable subpopulation enriched for Y-sperm comprising at least 72% Y-sperm.

5.   A viable subpopulation according to either of claims 3 or 4 having been sorted according to the method of claim 1 or claim 2.

6.   X-enriched sperm-plasma-membrane vesicles.

7.   Y-enriched sperm-plasma-membrane vesicles.

8.   The vesicles according to claim 6 or 7 from cattle.

9.   The vesicles according to claim 6 or 7 from a species selected from the group consisting of dog, cat, horse, pig, and sheep.

10.   The vesicles according to claim 6 or 7 from humans.

11.   The vesicles according to claims 6 or 7 wherein the vesicles comprise at least 80% head plasma membrane.

12.   X-enriched non-membrane sperm component.

13.   Y-enriched non-membrane sperm component.

14.   The component according to claim 12 or 13 from cattle.

15.   The component according to claim 12 or 13 from a species selected from the group consisting of dog, cat, horse, pig, and sheep.

16.   The component according to claim 12 or 13 from humans.

17.　　A refined X sex-associated membrane protein.

18.　　A substantially pure X sex-associated membrane protein.

19.　　The protein according to claim 18 selected from group consisting of proteins having a molecular weight and pI as follows:

　　　　(1)　　20.9, 5.74;
　　　　(2)　　26.3, 7.58;
　　　　(3)　　27.8, 6.08;
　　　　(4)　　44.1, 6.90;
　　　　(5)　　52.5, 5.33;
　　　　(6)　　58.0, 5.99;
　　　　(7)　　59.4, 6.59;
　　　　(8)　　59.5, 6.81;
　　　　(9)　　62.1, 7.23;
　　　(10)　　62.5, 5.54;
　　　(11)　　62.7, 6.85;
　　　(12)　　62.8, 6.64;
　　　(13)　　63.9, 5.83;
　　　(14)　　68.2, 5.95; and
　　　(15)　　78.6, 7.14.

20　　A refined Y sex-associated membrane protein.

21.　　A substantially pure Y sex-associated membrane protein.

22.　　The protein according to 21 selected from the group consisting of proteins having a molecular weight and pI as follows:

　　　　(1)　　9.6, 6.58;
　　　　(2)　　19.9, 5.67;
　　　　(3)　　29.0, 6.67;

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(4)   36.5, 7.16;
(5)   41.1, 6.21;
(6)   55.5, 6.82;
(7)   55.9, 5.25;
(8)   58.0, 8.67;
(9)   62.9, 6.34; and
(10) 70.3, 5.77.

23.   An antibody which binds to a mammalian
X sex-associated membrane (X-SAM) protein, said anti-
body being essentially free of antibodies which bind
to a mammalian Y sex-associated membrane protein, to
the H-Y antigen, or to non-sex-specific components
of the sperm plasma membrane.

24.   An antibody which binds to a mammalian
Y sex-associated membrane (Y-SAM) protein, said anti-
body being essentially free of antibodies which bind
to a mammalian X sex-associated membrane protein, to
the H-Y antigen, or to non-sex-specific components
of the sperm plasma membrane.

25.   The antibody of claim 23 or 24 wherein
said antibody is a monoclonal antibody.

26.   The antibody of claim 23 or 24
wherein said X-SAM protein is from cattle.

27.   The antibody of claim 23 or 24 wherein
said X-SAM protein is from a species selected from
the group consisting of dog, cat, horse, pig, or
sheep.

28.   The antibody of claim 23 or 24 wherein
said X-SAM protein is from humans.

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29.   A semen sample enriched for mammalian Y-sperm comprising a semen sample incubated with the antibody of claim 23 under conditions appropriate to inactivate X-sperm.

30.   A semen sample enriched for mammalian X-sperm comprising a semen sample incubated with the antibody of claim 24 under conditions appropriate to inactivate Y-sperm.

31.   The semen sample of claim 29 or 30 wherein said conditions include incubation in phosphate buffer, pH 6.4 - pH 8.4 at 37°C for at least 15 min with an excess of antibody.

32.   A method for increasing the probability that a mammalian offspring will be male comprising the step allowing a sperm from the semen sample of claim 29 to fertilize an ovum.

33.   A method for increasing the probability that a mammalian offspring will be female comprising the step allowing a sperm from the semen sample of claim 30 to fertilize an ovum.

34.   A method for increasing the probability that a mammalian offspring will be female comprising the step of immunizing a female mammal with a Y-SAM protein.

35.   A method for increasing the probability that a mammalian offspring will be male comprising the step of immunizing a female mammal with an X-SAM protein.

36.   A method for decreasing the fertility of a female mammal comprising the step of immunizing the female with both X- and Y-SAM proteins.

37.   The method of any one of claims 32-36 wherein the mammal is a cattle.

38.   The method of any one of claims 32-36 wherein the mammal is selected from the group consisting of dog, cat, horse, sheep, and pig.

39.   The method of any one of claims 32-36 wherein the mammal is a human.

40.   An artifical insemination kit comprising a straw containing the semen sample of claim 29 and a cryopreservative.

41.   An artifical insemination kit comprising a straw containing the semen sample of claim 30 and a cryopreservative.

42.   The kit of claim 40 or 41 wherein the semen sample is from cattle.

43.   A method of detecting in a sample, antibodies which bind to a SAM protein comprising the steps of

(a)   contacting the SAM protein with the sample to create a mixture

(b)   contacting the mixture with a detectable antibody whch binds to immuno-globulin molecules.

44.   A method of increasing the probability that a mammalian offspring will carry a gene for a

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particular Y-chromosome linked trait or decreasing
the probability that a mammalian offspring will carry
a gene for a particular X-chromsome-linked trait
comprising the step of allowing a sperm from the
semen sample of claim 29 to fertilize an ovum.

45.   A method of increasing the probability
that a mammalian offspring will carry a gene for a
particular X-chromosome linked trait or decreasing
the probability that a mammalian offspring will
carry a gene for a particular Y-chromsome-linked
trait comprising the step of allowing a sperm from
the semen sample of claim 30 to fertilize an ovum.

AMENDED CLAIMS
received by the International Bureau
on 12 September 1990 (12.09.90);
original claims 2,22,27,29,30,32,and 33 amended;
other claims unchanged (7 pages)]

1. A method of sorting living sperm cells whose DNA content difference is greater than 1% comprising the steps of:

(1) staining living sperm cells with a dye which, in response to laser excitation, emits fluorescent light in proportion to the amount of DNA in the cell; and

(2) sorting the sperm cells according to their fluorescence with a flow cytometer into subpopulations enriched in X-chromosome-bearing sperm cells and Y-chromosome-bearing sperm cells, said flow cytometer

(a) having a signal to noise ratio of at least 100:1,

(b) delivering a laser power to each cell of at least 90 mW,

(c) having a PMT quantum efficiency of at least 8%, and

(d) being capable of adjusting the sperm cells into the same orientation.

2. The method according to claim 1, wherein the signal to noise ratio is at least 1000:1, the laser power delivered to each cell is at least 160 mW, and the PMT quantum efficiency is at least 20%.

3. A viable subpopulation enriched for X-sperm comprising at least 68% X-sperm.

4. A viable subpopulation enriched for Y-sperm comprising at least 72% Y-sperm.

5.   A viable subpopulation according to either of claims 3 or 4 having been sorted according to the method of claim 1 or claim 2.

6.   X-enriched sperm-plasma-membrane vesicles.

7.   Y-enriched sperm-plasma-membrane vesicles.

8.   The vesicles according to claim 6 or 7 from cattle.

9.   The vesicles according to claim 6 or 7 from a species selected from the group consisting of dog, cat, horse, pig, and sheep.

10.   The vesicles according to claim 6 or 7 from humans.

11.   The vesicles according to claims 6 or 7 wherein the vesicles comprise at least 80% head plasma membrane.

12.   X-enriched non-membrane sperm component.

13.   Y-enriched non-membrane sperm component.

14.   The component according to claim 12 or 13 from cattle.

15.   The component according to claim 12 or 13 from a species selected from the group consisting of dog, cat, horse, pig, and sheep.

16. The component according to claim 12 or 13 from humans.

17. A refined X sex-associated membrane protein.

18. A substantially pure X sex-associated membrane protein.

19. The protein according to claim 18 selected from group consisting of proteins having a molecular weight and pI as follows:
  (1)   20.9, 5.74;
  (2)   26.3, 7.58;
  (3)   27.8, 6.08;
  (4)   44.1, 6.90;
  (5)   52.5, 5.33;
  (6)   58.0, 5.99;
  (7)   59.4, 6.59;
  (8)   59.5, 6.81;
  (9)   62.1, 7.23;
  (10) 62.5, 5.54;
  (11) 62.7, 6.85;
  (12) 62.8, 6.64;
  (13) 63.9, 5.83;
  (14) 68.2, 5.95; and
  (15) 78.6, 7.14.

20. A refined Y sex-associated membrane protein.

21. A substantially pure Y sex-associated membrane protein.

22. The protein according to claim 21 selected from the group consisting of proteins having a molecular weight and pI as follows:

    (1)   9.6, 6.58;
    (2)   19.9, 5.67;
    (3)   29.0, 6.67;
    (4)   36.5, 7.16;
    (5)   41.1, 6.21;
    (6)   55.5, 6.82;
    (7)   55.9, 5.25;
    (8)   58.0, 8.67;
    (9)   62.9, 6.34; and
    (10) 70.3, 5.77.

23. An antibody which binds to a mammalian X sex-associated membrane (X-SAM) protein, said antibody being essentially free of antibodies which bind to a mammalian Y sex-associated membrane protein, to the H-Y antigen, or to non-sex-specific components of the sperm plasma membrane.

24. An antibody which binds to a mammalian Y sex-associated membrane (Y-SAM) protein, said antibody being essentially free of antibodies which bind to a mammalian X sex-associated membrane protein, to the H-Y antigen, or to non-sex-specific components of the sperm plasma membrane.

25. The antibody of claim 23 or 24 wherein said antibody is a monoclonal antibody.

26. The antibody of claim 23 or 24 wherein said X-SAM protein is from cattle.

27. The antibody of claim 23 or 24 wherein said X-SAM protein is from a species selected from the group consisting of dog, cat, horse, pig, and sheep.

28. The antibody of claim 23 or 24 wherein said X-SAM protein is from humans.

29. A mammalian semen sample enriched for Y-sperm comprising viable Y-sperm and X-sperm substantially inactivated with the antibody of claim 23.

30. A mammalian semen sample enriched for X-sperm comprising viable X-sperm and Y-sperm substantially inactivated with the antibody of claim 24.

31. The semen sample of claim 29 or 30 wherein said conditions include incubation in phosphate buffer, pH 6.4 - pH 8.4 at 37°C for at least 15 min with an excess of antibody.

32. A method for increasing the probability that a mammalian offspring will be male comprising the step of allowing a sperm from the semen sample of claim 29 to fertilize an ovum.

33. A method for increasing the probability that a mammalian offspring will be female comprising the step of allowing a sperm from the semen sample of claim 30 to fertilize an ovum.

34. A method for increasing the probability that a mammalian offspring will be female comprising

the step of immunizing a female mammal with a Y-SAM
protein.

35. A method for increasing the probability
that a mammalian offspring will be male comprising the
step of immunizing a female mammal with an X-SAM
protein.

36. A method for decreasing the fertility of
a female mammal comprising the step of immunizing the
female with both X- and Y-SAM proteins.

37. The method of any one of claims 32-36
wherein the mammal is a cattle.

38. The method of any one of claims 32-36
wherein the mammal is selected from the group
consisting of dog, cat, horse, sheep, and pig.

39. The method of any one of claims 32-36
wherein the mammal is a human.

40. An artifical insemination kit comprising
a straw containing the semen sample of claim 29 and a
cryopreservative.

41. An artifical insemination kit comprising
a straw containing the semen sample of claim 30 and a
cryopreservative.

42. The kit of claim 40 or 41 wherein the
semen sample is from cattle.

43. A method of detecting in a sample, antibodies which bind to a SAM protein comprising the steps of

(a) contacting the SAM protein with the sample to create a mixture

(b) contacting the mixture with a detectable antibody whch binds to immunoglobulin molecules.

44. A method of increasing the probability that a mammalian offspring will carry a gene for a particular Y-chromosome linked trait or decreasing the probability that a mammalian offspring will carry a gene for a particular X-chromsome-linked trait comprising the step of allowing a sperm from the semen sample of claim 29 to fertilize an ovum.

45. A method of increasing the probability that a mammalian offspring will carry a gene for a particular X-chromosome linked trait or decreasing the probability that a mammalian offspring will carry a gene for a particular Y-chromsome-linked trait comprising the step of allowing a sperm from the semen sample of claim 30 to fertilize an ovum.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 39/395   35/52; 37/02
U.S. Cl.: 424/85.9; 105

II. FIELDS SEARCHED

Minimum Documentation Searched 7	
Classification System	Classification Symbols
U.S.	424/105; 85.9 530/852
	435/2; 808 514/2; 8

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

Chemical Abstracts 11th collective vol 96-105 (1982-1986)
"Sperm"

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y A	US, A, 4,722,887 (FABRICANT) 2 February 1988 See entire doucment	3-5 1-2 and 6-45
Y A	US, A, 4,448,767 (BRYANT) 15 May 1984 See entire doucment	3-5 1-2 and 6-45
A	Chemical Abstracts Vol. 100 issued 1984 Yan et al. "Monoclonal Antibody" page 448 abstract 66351e Am. J. Reprod. Immunol. 1983 4(3) 111-15 (Eng)	23-33

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8 August 1989	11 OCT 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	Sam Rosen

Form PCT/ISA/210 (second sheet) (Rev.11-87)

Category	Citation of Document. with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 103 Issued 1985, Hunt et al "Isolation of Major Proteins", page 371 abstract 119,351a, Prep. Biochem. 1985, 15 (1-2) pages 9-33 (Eng).	17-22
A	Chemical Abstracts volume 103 issued 1985 Peterson et al., "Further characterization of boar sperm plasma membrane protein" page 261 abstract 191643q, Gamete Res. 1985, 12(1) pages 91-10U (Eng)	6-22
A	Chemical Abstracts volume 103 issued 1985 Saji et al "A human sperm" page 547 abstract 139897m Am. J. Reprod Immunol, Microbiol., 1985 8(4) pages 132-6 (Eng)	17-22
A	Chemical Abstracts volume 98 issued 1983 Peterson et al., "Characterization of boar sperm plasma membranes" page 356 abstract 175817x J. Androl. 1983, 4(1) pages 71-81 (Eng)	12-22
A	Chemical Abstracts volume 104 issued 1986 Braun et al "Method and apparatus for Fluorometric Determination" page 282 abstract 17352e Ger.(East) DD222,123 (1984)	1-2

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)